Journal of Food Composition and Analysis 34 (2014) 153–162

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original Research Article

A simple liquid chromatography coupled to quadrupole time of flight mass spectrometry method for macrolide determination in tilapia fillets

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ARTICLE INFO

Article history:
Received 5 March 2013
Received in revised form 5 February 2014
Accepted 6 February 2014

Keywords:
Food analysis
Veterinary drug residues
Food safety
Food contaminants
Antimicrobial residues
Macrolides
Liquid chromatography
Mass spectrometry
LC–QToF
Aquaculture
Tilapia

ABSTRACT

A method for the identification and quantification of macrolides (erythromycin, josamycin, tilmicosin, tylosin, spiramycin and neospiramycin) in tilapia fillets by liquid chromatography coupled to quadrupole time of flight mass (LC–QToF) spectrometry is presented. Sample preparation was quite simple and low cost: proteins were precipitated and the analytes were extracted with ethanol, extract was defatted with hexane and concentrated by solvent evaporation. The matrix effect was statistically demonstrated during method validation, in which matrix-matched calibration was applied. Matrix effect mechanism was clarified thanks to the capability of QToF mass spectrometer of generating full scan spectra with accurate mass measurement. The limits of quantification were at least 45% lower than the maximum residue limits. The method was able to identify the studied macrolides with relative *m/z* errors lower than 2.5 ppm and to monitor two fragment ions per analyte, which is in accordance with the European Community recommendations for the analysis of contaminants in foods. Samples from the retail market of São Paulo State, Brazil, were analyzed by the developed method and none of them presented positive results for the macrolides studied.

1. Introduction

World fish capture reached its apex in the 1990s. In contrast, aquaculture has presented growth rates close to those of the global population (FAO, 2009). Tilapia is the most widely raised species of any farmed fish. In 2004, tilapia was the eighth most popular seafood in the USA, and its production was projected to increase from 1.5 million tons in 2003 to 2.5 million tons in 2010, with a sales value of more than USD 5 billion (FAO, 2010). In this context, tilapia farming presents a very competitive scenario where producers keep the maximum fish density within a single tank. The use of antimicrobials in the production system for therapeutic (disease control) and prophylactic (disease prevention) purposes is almost inevitable because the spread of an eventual bacterial disease in the production tanks would certainly jeopardize production.

In the production of foods of animal origin, the incorrect use of antimicrobials or the disrespect of withdrawal time after treatment can lead to the presence of antibiotic residues in foods. These residues can promote the growth of pathogenic, drug-resistant bacterial strains and also cause allergic reactions in some hypersensitive individuals (Wang, 2009).

Macrolides are highly potent antimicrobials used in veterinary practices against a wide variety of Gram-positive and Gram-negative bacteria. Chemically, they consist of macrocyclic lactone rings with 14 (erythromycin, roxithromycin and clarithromycin), 15 (azithromycin) or 16 (spiramycin, tylosin, tilmicosin and josamycin) carbons linked to the carbohydrate molecules, presenting lipophilic and basic characteristics (Kanfer et al., 1998). In general, macrolide antibiotics present pK_a values between 7.1 and 9.9 (Gobel et al., 2004). These are important characteristics when someone considers the development of methods for the extraction of these substances from food matrices

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http://dx.doi.org/10.1016/j.jfca.2014.02.006
0889-1575/

and their chromatographic separation. Some macrolide antibiotics are sensitive to low pH and suffer degradation under acidic conditions (Horie, 1995). Fig. 1 illustrates the molecular structures of the macrolides studied in this work.

The World Organization for Animal Health (OIE, 2007) considers three macrolides (josamycin, erythromycin and spiramycin) as "critically important" antimicrobials for fish harvesting because they are essential for fighting against specific infections that affect some species and because there is a lack of therapeutic alternatives.

Codex Alimentarius (2009) including the regional (European Community (EMA, 2011; and MERCOSUR, 2000) and national regulatory agencies (USDA, 2010; MAPA, 2010; and JFCRF, 2010) have all established maximum residue limits (MRL) for macrolides in food matrices, and some of them include MRL for fish matrices. Nonetheless, it is important to notice that neither the MERCOSUR (2000), nor the USFDA (2011) or Brazilian (MAPA, 2010) regulatory agencies have approved any macrolide drug for use in aquaculture.

The presence of macrolide residues in food due to their abusive use in veterinary practices has a significant impact on public health and on food international trade, and this has raised concerns in the scientific community and the regulatory agencies (Wang, 2009). Analytical methods for the identification and quantification of these antimicrobials are fundamental for the establishment of effective dose recommendations and withdrawal periods through pharmacokinetic studies and to evaluate the impact of the employment of these substances in aquaculture from an economic and food safety perspective.

Numerous analytical methods for the determination of residues of macrolides in fish and other edible animal tissues have been published recently (Jo et al., 2011; Horie et al., 2003; Wang and Leung, 2007; Lucchetti et al., 2005). However, few scientific papers about the analytical methods for quantifying macrolides in tilapia fillets by the use of liquid chromatography coupled to quadrupole time of flight (LC–QToF) spectrometry have been reported. Mass spectrometry (MS) is a universal detection technique, which is ideal for multi-residue analysis. The most commonly mass analyzers applied for contaminant determination in food matrices are triple-quadrupoles (QqQ). Although QqQ analysers present better sensitivity, QToF mass spectrometers can obtain full scan spectra with medium to high resolution and accurate mass measurement, besides having the capability of performing MS/MS analysis, so they generate spectra of better qualitative information, with enough sensitivity to meet the target of quantifying contaminants at concentrations below their maximum residue levels.

This article presents a simple method for the identification and quantification of six macrolides (erythromycin, josamycin, tilmicosin, tylosin, spiramycin and neospiramycin) in tilapia fillets by liquid chromatography coupled to a hybrid mass spectrometry system composed of quadrupole time of flight mass analyzers (LC–QToF). After validation, the method was applied to analyze samples from the retail market of São Paulo State, Brazil.

2. Materials and methods

2.1. Samples

Blank samples of refrigerated tilapia fillets were supplied by the School of Agronomical and Veterinary Sciences, Julio de Mesquita State University (UNESP – Jaboticabal, SP, Brazil), which guaranteed the absence of macrolide residues. These samples were separated into portions of approximately 500 g and stored at -25 ± 2 °C before being analyzed. The analytical signal of the tested macrolides proved to be stable for a period of ten days when ground, blank tilapia fillets were fortified with them at MRL concentrations and stored at -25 ± 2 °C.

Twenty samples were acquired from the retail market of São Paulo State (Brazil) and stored at -25 ± 2 °C before analysis. Twelve of the samples were entire fish obtained from "fish and pay pond" establishments (from the cities of Espirito Santo do Pinhal, Batatais and Ribeirão Preto) and were filleted before freezing, four were frozen fillets from large chain supermarkets (from the city of Campinas), and four were refrigerated, whole fish obtained from street fairs (from cities of Campinas, Jaguariuna and São Paulo), which were also filleted before freezing. All the samples were analyzed within a maximum period of 10 days of storage.

2.2. Chemicals and reagents

Reagent grade ethanol (Synth, Brazil) and LC grade n-hexane (Omnisolv, USA) were used in the extraction and clean up processes. LC grade methanol (MeOH) (Burdick & Jackson, USA), glacial acetic acid (HAc) (Merck, Brazil), and deionized water (purified by a Gehaka OS20 LX System, Brazil) were used to compose the mobile phase for liquid chromatography.

Primary–secondary amine (PSA), florisil, neutral aluminum oxide, and octadecilsilane (C_{18}) (Sigma–Aldrich, Germany) were tested as dispersive solid phase extraction (DSPE) and matrix solid-phase dispersion (MSPD) adsorbents.

Polyvinylidene difluoride (PVDF) hydrophilic and polytetrafluorethylene (PTFE) membranes (Millipore – USA) with 0.22 μm pore size were used to filter the aqueous and organic mobile phase solutions, respectively. PVDF hydrophilic syringe filters (Millipore – USA), with a 33 mm diameter and 0.22 μm pore size, were applied to filter the sample extracts before chromatographic injection.

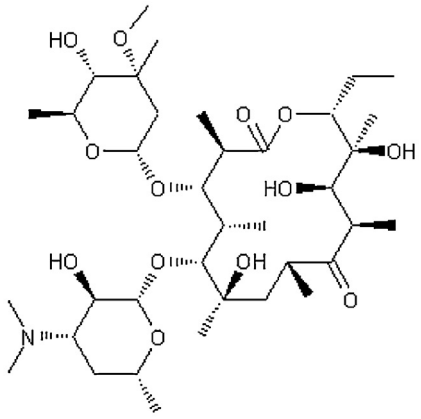
Macrolide analytical standards used in this study were: erythromycin A (96.7%, U.S. Pharmacopeia, USA); roxithromycin (97.6%, Sigma–Aldrich, Switzerland); neospiramycin I (97.7%, Waco, Japan); spiramycin (88.9%, Fluka, Germany); tilmicosin (86.5%, Fluka, Germany); josamycin (100%, Fluka-Biochemika, Japan) and tylosin tartrate (98.0%, Dr. Ehrenstofer, Germany).

2.3. Standard solutions

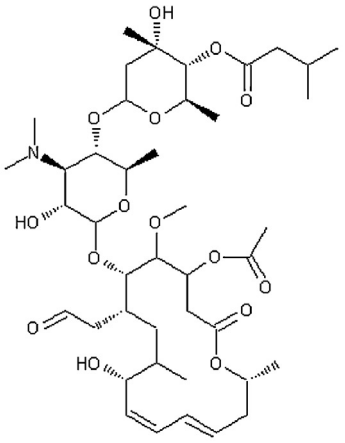
All standard solutions were prepared in LC grade methanol. Stock solutions were prepared at the concentration of 1000 μg mL^{-1} and stored in tightly closed amber vessels at -25 °C for a maximum period of 3 months. Working solutions were prepared daily as a mixture of all macrolides through the dissolution of stock solutions and were used immediately after preparation. The final concentrations of macrolides in the working solutions were: 1.2 μg mL^{-1} for erythromycin; 4.0 μg mL^{-1} for spiramycin and neospiramycin; 2.0 μg mL^{-1} for tylosin and 1.0 μg mL^{-1} for tilmicosin and josamycin. The roxithromycin working solution was prepared separately at the concentration of 4.0 μg mL^{-1} and used as an internal standard. It was observed that the analytical signal of the stock solutions remained stable for a period of approximately 4 months when stored in tight closed amber vessels at -25 °C.

2.4. Equipment

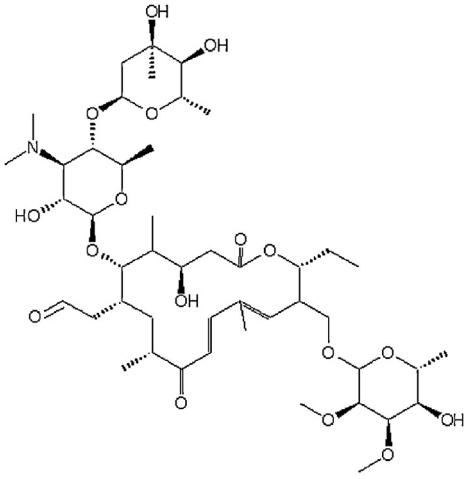
A BL 2105 analytical balance (Sartorius, Germany) was used to weigh the reagents and standards. Samples were ground with a Walita RI 2044 mini food processor (Philips, Brazil). An Ultrasonic Cleaner (Cole Parmer, USA), a Himac CR21 centrifuge (Hitachi, Japan), an AP56 Vortex Agitator (Phoenix, Brazil) and a Centrivap



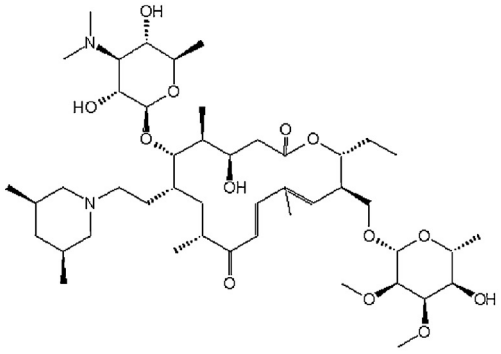
Erythromycin A



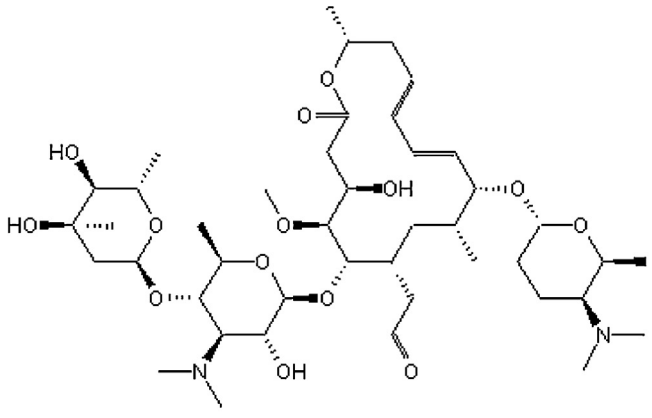
Josamycin



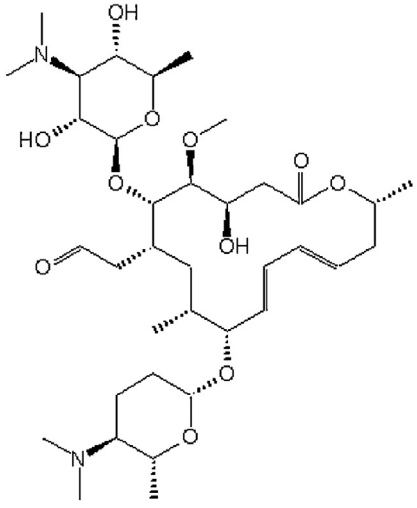
Tylosin A



Tilmicosin



Spiramycin I



Neospiramycin I

Fig. 1. Molecular structure of the studied macrolides.

Centrifuge Concentrator (Labconco, USA) were employed in sample preparation.

Liquid Chromatography was performed using a Waters Alliance 2695® LC system (Waters, USA), composed of quaternary pumping and automatic injection systems. The LC and MS systems were interfaced by an electrospray ionization (ESI) source. A hybrid QToF Micro® (Micromass, UK) mass spectrometer, composed of quadrupole (Q) and time of flight (ToF) analyzers with a hexapole collision cell (with helium as the collision gas), was employed to obtain full scan and product ion mass spectra (MS/MS), with an minimum resolution of 5000 FWHM. The LC–MS system control and data acquisition was performed using MassLynx 4.0 software (Micromass, UK).

2.5. Sample preparation

Sample preparation was carried out by the alcoholic precipitation of tilapia fillet proteins, with the simultaneous extraction of the analytes. Four grams of ground tilapia fillet portions were placed in 30 mL centrifuge tubes and submitted to ultra-sound bath for 5 min before the addition of 5 mL of reagent grade ethanol. The mixtures were homogenized in a vortex mixer for 1 min and then centrifuged at 3500 × *g* for 15 min. Supernatants were transferred to new 30 mL centrifuge tubes, and another 5 mL of ethanol were added to the precipitates, which were homogenized and centrifuged under the same conditions. The combined supernatants resulted in approximately 13 mL of turbid extracts. The extracts were then centrifuged at 1300 × *g* for 5 min to precipitate the debris, and 5 mL aliquots of the supernatant were transferred into 30 mL round bottom glass centrifuge tubes. Cleanup was performed by adding 3 mL of LC grade n-hexane followed by vortex agitation for 30 s. The upper phases (n-hexane) were discarded by a vacuum tube after phase separation. The defatted extracts were dried in a centrifuge concentrator at 60 °C, suspended in 1 mL of H_2O:MeOH (8:2, v/v), filtered through 0.22 μm pore size syringe filters and injected into the LC system (20 μL per injection). A schematic representation of the extraction procedure is shown in Fig. 2.

During the initial tests for extraction, deproteinization with acetonitrile was first evaluated. However, the extract formed a stable interface emulsion with n-hexane, interfering with the cleanup step and seriously affecting the repeatability of the method. Methanol presented protein precipitation and macrolides extraction properties similar to ethanol as extraction solvent. However, ethanol was chosen because it is a less expensive and less toxic solvent.

Dispersive solid phase extraction (DSPE) and matrix solid-phase dispersion (MSPD) with florisil, neutral aluminum oxide, octadecilsilane (C_{18}) and primary–secondary amine (PSA) as absorbents were also attempted. Nevertheless, the recoveries for macrolides were very poor when compared to the analytes diluted in water: methanol (8:2, v/v) at the same concentrations. These tests did not present satisfactory precision either.

All DSPE and MSPD tests were performed involving the same amount of sample (4 g) and methanol, acetonitrile and ethanol as solvents, at the same volume (10 mL) as the extraction procedure described above. DSPE was tested by vigorously mixing 100 mg of the adsorbent with the solvent extract of tilapia fillet spiked with the analytes at concentration levels corresponding to two times their respective MRL, in order to clean it up. MSPD was tried out by passing the solvent through syringe cartridges packed with 4 g of the adsorbent mixed with 4 g of the tilapia fillet spiked with the analytes at concentration levels corresponding to two times their respective MRL. The latter concentration by evaporation and suspension in solvent steps were the same as described above. When final extracts were injected in the LC–QToF system, the analytes peaks were quite small, with intensities close to 2 or at the most 3 times the base line noise. The analytes presented high affinity to the adsorbents used in DSPE and MSPD tests and none of the tested solvents were capable of withdrawing them from the active sites satisfactory. Acetic acid, added to the extraction solvent at the concentration of 10% (v/v), significantly improved macrolides recovery from the adsorbents, but macrolides (particularly erythromycin) rapidly degraded under this acidic condition.

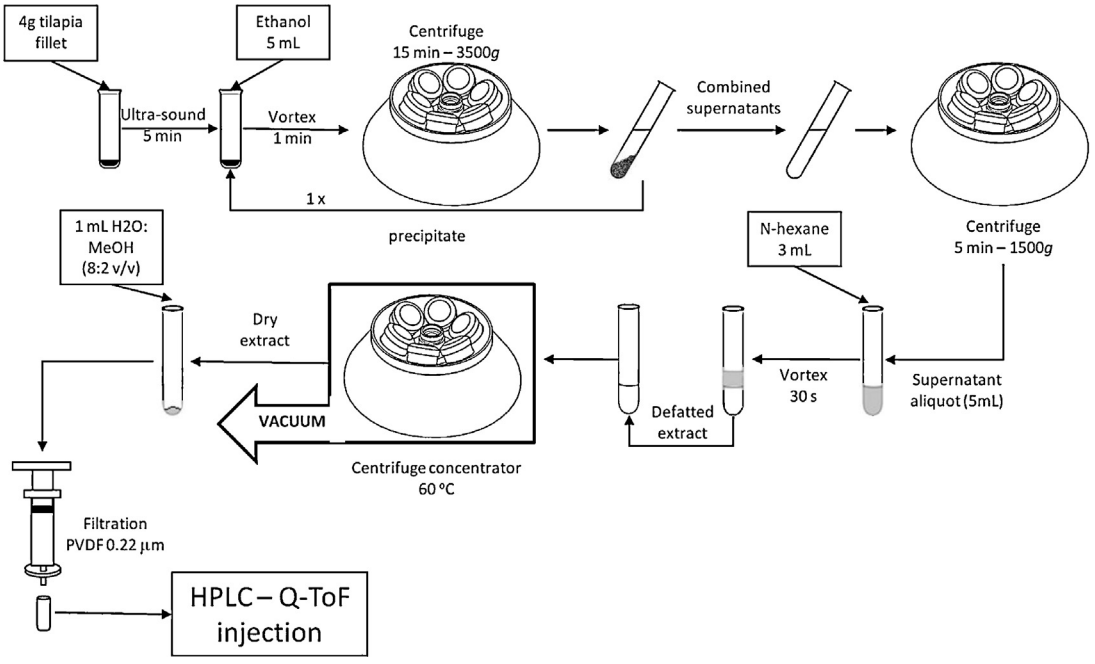


Fig. 2. Schematic representation of the macrolides extraction procedure from tilapia fillet.

2.6. LC–QToF conditions

Chromatographic separation was performed with a C_{18} XTerra® MS (150 mm × 2.1 mm, 5 μm – Waters, USA) reversed-phase column at 25 °C. Aqueous HAc 1% (v/v) (solvent A) and 1% HAc in methanol (solvent B) constituted the mobile phase. The following gradient profile at a constant flow rate of 0.3 mL min^{-1} was applied for the chromatographic separation: 0–2.7 min: isocratic 20% B; 2.7–4.0 min: convex gradient to 80% B (gradient curve number 3 – according to Waters Alliance® specifications); 4.0–10.5 min: isocratic 80% B; 10.5–16 min: concave gradient to 20% B (gradient curve number 11 – according to Waters Alliance® specifications).

Macrolide analytical signals were optimized at a probe voltage of 3 kV, an extraction cone at 2 V, source and desolvation temperatures of 130 °C and 380 °C, respectively, ion and collision energies of 0 V and 15 V, respectively and cone and desolvation gas flows at 50 and 440 L h^{-1}, respectively. Quantification and exact *m/z* measurements were performed on two channels in MS full scan mode, with an *m/z* range 826–945 at a cone voltage of 50 V for spiramycin, tylosin, tilmicosin and josamycin and an *m/z* range 697–864 at a cone voltage of 20 V for erythromycin, neospiramycin and roxithromycin (internal standard).

Because QToF analysers present significant sensitivity loss when operated at MS/MS mode, samples were submitted to a second chromatographic run after quantification under the same tuning conditions, except for the collision energy, which was increased to obtain the best tuning for the simultaneous detection of the precursor and at least two fragment ions for each macrolide, in order to achieve identity confirmation. The macrolide identity was confirmed when analyte retention time and MS/MS spectrum coincided with the analytical standard. Table 1 presents macrolides molecular formulas, monoisotopic masses, quantification ions, collision energies applied to obtain fragmentation spectra, confirmation ions and resulting identification points (IPs).

2.7. Method validation

The validation procedure was conducted by considering the macrolide MRLs adopted by the international regulatory and inspection agencies. After extensive literature research the following MRL values were adopted: 60 μg kg^{-1} for erythromycin (erythromycin A), 200 μg kg^{-1} for spiramycin (sum of spiramycin I and neospiramycin I), 50 μg kg^{-1} for josamycin in perciform fish (JFCRF, 2010), 50 μg kg^{-1} for tilmicosin in all food-producing species except poultry, and 100 μg kg^{-1} for tylosin (tylosin A) in all food-producing species (EMA, 2011).

Method validation was performed by considering the main international regulatory agencies, such as the European Community (EC, 2002) and the International Union of Pure and Applied Chemistry (IUPAC) (Thompson et al., 2002), as well as the Brazilian recommendations (MAPA, 2009). As the validation procedure for the developed method in this study was directed to a single-laboratory validation, the number of replicates at each concentration level for each analyte evaluated in all validation parameters was resumed to three ($n = 3$) in comparison to the replicates number ($n = 6$) suggested by MAPA (2009) and EC (2002).

Selectivity, analytical curve, linearity, sensitivity and detection and quantification limits were evaluated using spiked samples at 0.0, 0.5, 1.0, 1.5 and 2.0 times the MRL of each macrolide, as recommended by the European Commission (EC, 2002) and Brazil (MAPA, 2009).

The minimum square method was applied to obtain linear regression parameters for the calibration curves and to ascertain the analytical curve and linearity. Data were gathered using the Quanlynx® package (Micromass, UK), and statistical results were obtained using Microsoft Excel Software (Microsoft, USA). The calibration curve was plotted by applying the concentrations at the *x*-axis and the analyte area/internal standard area ratio at the *y*-axis.

Method selectivity was verified by the absence of interfering peaks that could possibly compromise the identification and integration of the analytes or the internal standard peaks in blank samples ($n = 10$).

Precision was determined in two conditions, using spiked samples at 0.5, 1.0 and 1.5 times the MRL of each macrolide: (i) intra-day precision: through the relative standard deviation (RSD) of the results of three replicates at each concentration level, analyzed on the same day by the same analyst and using the same instrument; and (ii) inter-day precision: through the RSD of the results of three replicates per day at each concentration level, analyzed by the same analyst, using the same instrument and performed on three different days.

Blank tilapia fillet samples were submitted to the sample preparation procedure to evaluate the accuracy and matrix effect. Final dry extracts were then spiked with analytical standard solutions before suspension in the water:methanol (8:2, v/v), employing the same concentration levels used in the precision tests.

Because no certified reference material (CRM) was available, accuracy was evaluated by performing recovery tests, analyzing blank samples that were spiked with the analytes before extraction (spiked samples) in three concentrations (0.5, 1.0 and 1.5 times the MRL; $n = 3$), and comparing them with blank samples that were spiked after extraction (spiked extracts) at the same concentrations ($n = 3$). The results were expressed as the percentage ratio between the absolute peak areas of the analytes (not divided by the internal standard area) of the spiked samples and the spiked extracts (EC, 2002; MAPA, 2009)

Matrix effect was evaluated at three concentrations (corresponding to 0.5, 1.0 and 1.5 times the MRL) by a comparison of the analyte signals in water:methanol solutions (8:2, v/v) with respect to those of spiked extracts, by *F* and *t* statistical tests ($\alpha = 5\%$), as recommended by the Brazilian guide (MAPA, 2009). Matrix effect was expressed as the percentage difference between the absolute

Table 1
Macrolides molecular formulas, monoisotopic masses, quantification ions, collision energies applied to obtain fragmentation spectra, confirmation ions and resulting identification points (IPs).

	Erythromycin A	Spiramycin I	Neospiramycin I	Tylosin	Tilmicosin	Josamycin
Molecular formula	$C_{37}H_{67}NO_{13}$	$C_{43}H_{74}N_2O_{14}$	$C_{36}H_{62}N_2O_{11}$	$C_{46}H_{77}NO_{17}$	$C_{46}H_{80}N_2O_{13}$	$C_{42}H_{69}NO_{15}$
Monoisotopic mass (Da)	733.4612	842.5140	698.4354	915.5192	868.5660	827.4667
Quantification ion (*m/z*) [*m/z* errors in ppm]	734.4691 [0.4]	843.522 [−1,3]	699.443 [1.3]	916.527 [−2.5]	869.5739 [−0.5]	828.4745 [0.5]
Collision energy (V)	20	30	20	30	40	25
IPs[a]: quantification ion	1	1	1	1	1	1
Identification ions (*m/z*)	576; 158	540; 174	540; 174	772; 174	696; 174	600; 174
IPs [a]: identification ions	3.0	3.0	3.0	3.0	3.0	3.0
TOTAL IPs[a]	4	4	4	4	4	4

[a]According to European Commission criteria (EC, 2002).

peak areas of the spiked extracts of the analytes, and those of the analytical standard solutions of the analytes in water:methanol (8:2, v/v).

The decision limit (CCα) and detection capacity (CCβ) of each macrolide were obtained from their respective absolute standard deviation, which was obtained from the inter-day precision test, as described by the EC (2002) and MAPA (2009). The CCα (α = 5%) was expressed as the MRL concentration plus 1.64 times the standard deviation of the intra-day precision. The CCβ (β = 5%) was expressed as the concentration corresponding to the CCα value plus 1.64 times the inter-day precision standard deviation.

IUPAC guide recommends that limits of detection (LOD) and quantification (LOQ) parameters should be evaluated. The LOQs were estimated as ten times the standard errors of the *y* intercepts of each analyte analytical curve, divided by their correspondent slopes; the LODs were estimated as the LOQs divided by three, as recommended by Miller and Miller (1988) and cited by Ribani et al. (2004). The accuracies and precisions at the estimated LOQ concentrations were confirmed by the analysis of ten blank samples spiked at the estimated LOQ concentrations before extraction.

3. Results and discussion

Table 2 presents the method validation results. The developed LC–QToF method was suitable for the simultaneous identification and quantification of several macrolides (erythromycin, josamycin, tilmicosin, tylosin and spiramycin) in the tilapia fillets. The obtained validation results were in accordance with the EC (2002) recommendations.

Analytes were extracted with adequate accuracy (between 77% for neospiramycin at 200 μg kg^{-1} and 109% for erythromycin at 75 μg kg^{-1}) and precision (RSDs in the intra- and inter-day tests were lower than 15% for all macrolides at all tested concentrations).

The method presented adequate linearity, with correlation coefficients (*r*) of 0.99 for all analytes. Method selectivity was verified by the absence of interfering peaks in the extracted ion chromatograms of the blank sample extracts in the time intervals around the retention times of the analytes, as shown in Fig. 3.

The LOD and LOQ indicated that the developed method is adequate for the quantification and identification of the analytes in tilapia fillet samples at lower concentrations as compared to the adopted MRLs. The application ranges reached concentrations of at least 2.2 times lower than the MRL.

The mass spectra of the macrolides showed a sharp change in the presence of the tilapia fillet matrix. The matrix effect was significant (α = 5%) for all analytes except tylosin, which demonstrates the importance and necessity of matrix matched calibration curves to guarantee the reliability of the results. The use of a mass spectrometer capable of acquiring full mass spectra was important to clarify such matrix effects. All of the analytes presented a sodium adduct [M+Na$^+$] as the most intense ion in the absence of the matrix. Conversely, the protonated molecules [M+H$^+$] were the most intense in the presence of the matrix for all the macrolides studied. This partially explains the high matrix effects for erythromycin, spiramycin, neospiramycin (up to 351%), tilmicosin and josamycin. When matrix was present, the ratio of tylosin [M+H$^+$]/[M+Na$^+$] also changed, but the increment in its protonated molecule absolute signal was not enough to produce a significant matrix effect. Table 3 shows the relative intensities of the protonated macrolide molecules and the respective sodium adduct ions present both in pure solvent and in the matrix.

Wang and Leung published a study on the detection of six macrolides in eggs, milk and honey. The ultrahigh pressure liquid chromatography–quadrupole-time-of-flight-mass spectrometry (UHPLC–QToF) technique used in that study obtained detection limits between 0.1 and 1 mg kg^{-1}, which are adequate enough to meet the regulatory requirements for routine analysis, and this technique identified desmycosin (tylosin B) in honey, based on accurate mass measurement and the use of elemental composition resource. This study compared the performance of the method with respect to the linearity, precision and detection limits for high-performance liquid chromatography–triple quadrupole mass spectrometry (HPLC–QqQ) and UHPLC–QToF, and it demonstrated that the HPLC–QqQ technique has a wider linear range than UHPLC–QToF, even when an enhanced dynamic range was enabled during the latter technique. The HPLC–QqQ technique also showed better repeatability due to the larger number of points obtained along chromatographic peak acquisition (Wang and Leung, 2007).

It is estimated that detectability of ToF and QToF mass spectrometers is one to two orders of magnitude lower than the QqQ technique in the selected reaction monitoring (SRM) mode (Barcelo and Petrovic, 2007). Thus, the applicability of ToF or QToF

Table 2
Validation results of the developed LC–QToF method.

		Erythromycin A	Spiramycin I	Neospiramycin I	Tylosin	Tilmicosin	Josamycin	
MRL (μg kg^{-1})			60	200	200	100	50	50
Application range (μg kg^{-1})			27–120	82–400	54–400	33–200	22–100	17–100
Linearity (*r*)			0.994	0.993	0.997	0.996	0.992	0.995
Intraday precision (%)	*0.5 × MRL*		7	7	6	10	1	7
	1.0 × MRL		2	3	4	6	4	6
	1.5 × MRL		3	5	3	3	6	1
Interday precision (%)	*0.5 × MRL*		6	8	10	13	10	13
	1.0 × MRL		14	13	9	7	9	13
	1.5 × MRL		5	7	4	3	6	5
Matrix effect (%)	*0.5 × MRL*		22	149	344	n.s.	214	n.s.
	1.0 × MRL		14	118	351	n.s.	160	−18
	1.5 × MRL		13	86	299	n.s.	165	−9
Accuracy (%)	*0.5 × MRL*		96	88	82	80	97	91
	1.0 × MRL		100	84	77	83	85	100
	1.5 × MRL		109	88	77	84	95	86
LOD (μg kg^{-1})			9	27	18	11	7.3	5.8
LOQ (μg kg^{-1})			27	82	54	33	22	17
CCα (μg kg^{-1})			65	208	205	104	53	53
CCβ (μg kg^{-1})			70	216	210	108	55	56

n.s.: not significant at α = 5%.

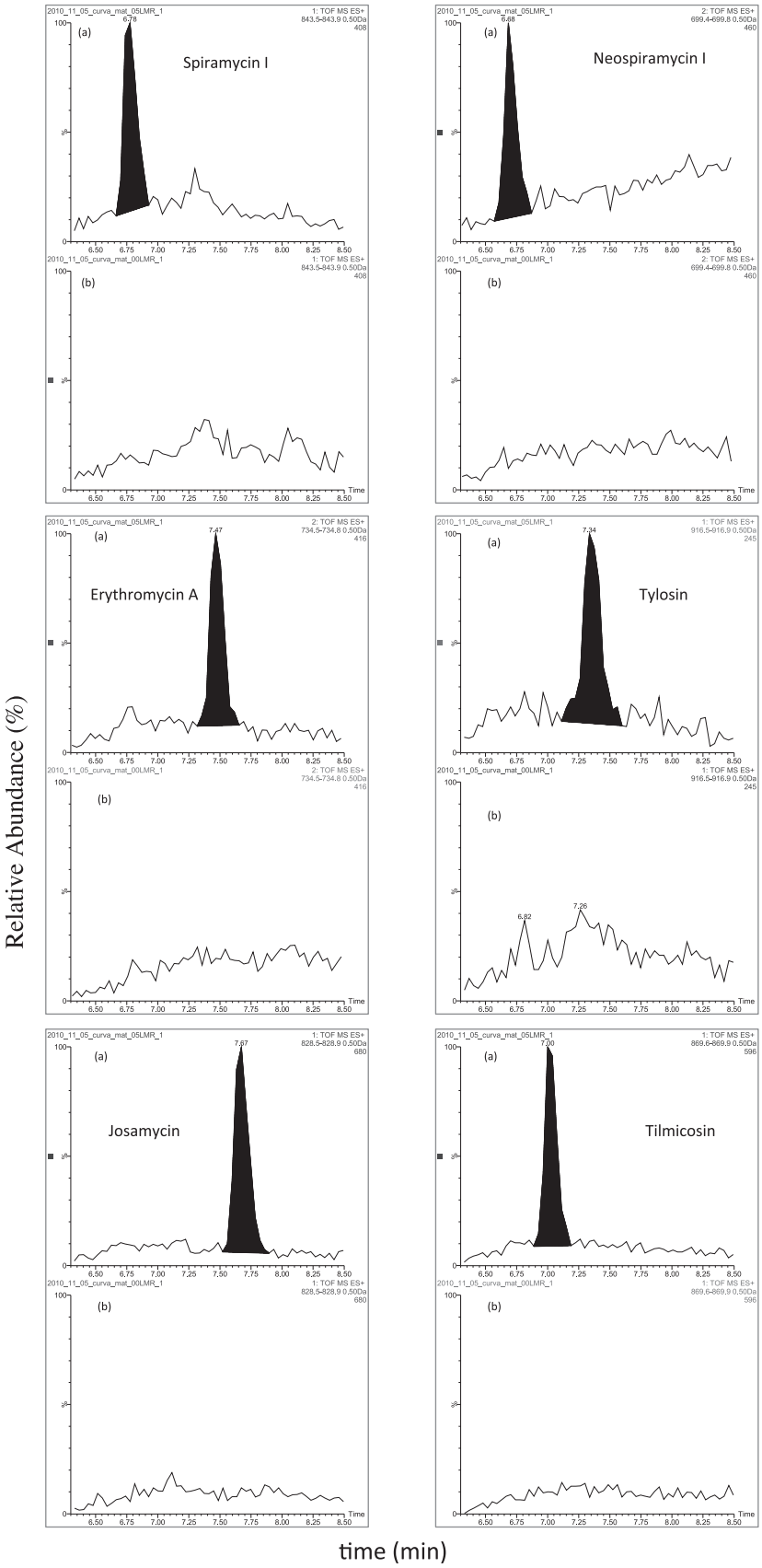


Fig. 3. (a) Example of extracted macrolides mass chromatograms in tilapia fillet matrix (0.5 × MRL concentrations) and (b) the respective blank tilapia fillet chromatograms.

Table 3
Relative intensities of protonated molecules and the respective sodium adduct ions ($[M+H]^+/[M+Na]^+$) in the presence and absence of tilapia fillet matrix.

Macrolide	Matrix present	Matrix absent
Spiramycin I	1.28	0.29
Neospiramycin I	1.38	0.25
Josamycin	1.21	0.83
Tilmicosin	7.39	4.17
Tylosin	1.71	0.58
Erythromycin A	1.03	0.53

mass spectrometry for the quantification of contaminants in food matrices is a challenge due to the low concentrations of these substances in food samples. Notably, the Wang and Leung (2007) study, involving the analysis by UHPLC–QToF, allowed for the determination of tylosin and the identification of its degradation product (desmycosin) in honey without requiring the use of the analytical standard tylosin B, which would be difficult to achieve with QqQ spectrometers.

Previous methods reported in literature for the determination of macrolides in fish matrices reinforce the effectiveness of the method presented in this study. Lucchetti et al. (2005) described a simple confirmatory method for the determination of erythromycin residues in trout by liquid chromatography coupled with triple quadrupole mass spectrometry. The method presented recoveries between 85 and 97% and CCα and CCβ values of 220 and 238 μg kg^{-1}, respectively (considering an MRL of 200 μg kg^{-1}), after sample deproteinization with acetonitrile, defatting with hexane and a linear range 0–1000 μg kg^{-1}, with recoveries in the range of 85.0 ± 9.2% at 100 μg kg^{-1}. In spite of the larger linear range (which is typical for QqQ in comparison with QToF mass spectrometers), the intra-day precision at 100 μg kg^{-1} presented a value approximately five and three times higher than the value presented in our study for this analyte, at 60 μg kg^{-1} and 90 μg kg^{-1}, respectively.

Horie et al. (2003) also reported a method for the determination of macrolide antibiotics (erythromycin, oleandomycin, kitasamycin, josamycin, mirosamicin, spiramycin, tilmicosin and tylosin) in meat and fish by liquid chromatography–single quadrupole mass spectrometry, which monitored at least 2 ions per analyte. Macrolides were extracted with 0.2% metaphosphoric acid:methanol (6:4, v/v), and the extracts were purified on an Oasis HLB cartridge (60 mg); the recoveries varied from 70.4 to 93.2%, and the LOQ was 10 μg kg^{-1}. Although this method presented better LOQ, the sample preparation procedure was more tedious and expensive because it involved the expense of a higher quantity of solvent (100 mL per sample) for the extraction and the filtering and solid phase extraction steps before sample concentration, and this still did not present recoveries higher than those obtained in this work. It should be emphasized that this method was not capable of performing MS/MS analysis for identity confirmation of the analytes.

Recently, Jo et al. (2011) also reported a method for the simultaneous determination of macrolide residues (erythromycin A, kitasamycin, josamycin, roxithromycin, tylosin A, and oleandomycin) in fish (flounder) and shrimp by LC–QqQ. The method presented limits of detection and quantification around one to two orders of magnitude lower than the presented study, which is expected for QqQ spectrometers, as mentioned above. It also presented similar recoveries and precision results, although sample preparation included more cleaning steps and a higher usage of solvents (approximately 300 mL of solvent per sample), corroborating the simplicity and the low cost of the sample preparation method introduced in this paper.

According to the EC (2002), confirmatory methods shall provide information about the chemical structure of the analyte, and MS detection is recognizably capable of accomplishing that. When the mass fragments are measured using methods other than full-scan techniques, a system of identification points (IPs) shall be used to interpret the data. For the confirmation of prohibited substances, a minimum of 4 identification points is required, and for the confirmation of permitted substances, a minimum of 3 identification points is required. Although QToF Micro® provides *m/z* accuracies with relative *m/z* errors of less than 5 ppm, its resolution is less than 10,000 and does not adhere to the EC criteria for high resolution mass spectrometry. Hence, at least 3 ions must be

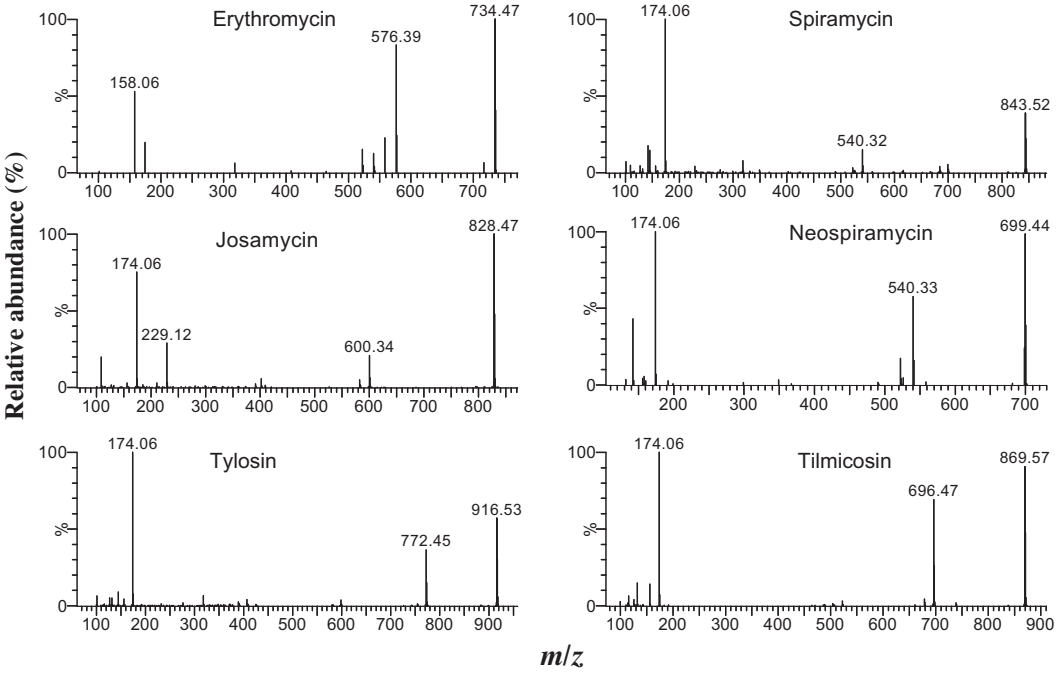


Fig. 4. Macrolides fragmentation spectra.

monitored in MS/MS mode to reach 3 identification points. In confirmatory separate runs, the method allowed the monitoring of the protonated molecules and two fragment ions for each analyte, confirming their identity according to the EC (2002) recommendations. Table 1 describes the quantification and confirmation ions monitored in the validated method, and the resulting IPs for each analyte. Fig. 4 presents the fragmentation spectra obtained for each macrolide studied. No lock mass correction was applied to obtain the data in Table 1 or to generate the spectra in Fig. 4.

It should be noted that the EC criteria for the identity confirmation of contaminants in food should be revised to include mass accuracy parameters. Hernández et al. (2004) highlighted a problem that arose with the EC, 2002/657 definition of "high resolution mass spectrometry instruments". This definition does not consider an important parameter for the confirmation of a chemical contaminant, mass accuracy, and it ignores the advantages of instruments with accurate mass capabilities, even if not all of them meet the "high-resolution" definition of the decision. Wang and Leung (2007) proposed alternative criteria for IP assignment, where relative *m/z* errors of less than 2 ppm would lead to 2 IPs per ion; relative *m/z* errors between 2 ppm and 10 ppm would result in 1.5 IPs per ion, and ions with more than 10 ppm of relative *m/z* error would render 1 IP. Following these criteria, the monitoring of the protonated molecular precursor ion and only one fragment ion would be necessary to obtain the required IPs for analyte confirmation with QToF Micro®.

Samples from the retail market of São Paulo State (Brazil) were analyzed by the LC–QToF validated method to test its applicability. None of the analyzed samples presented positive results that were higher than the limits of detection for the macrolides studied. Nevertheless, it is necessary to highlight that the number of tested samples was small ($n = 20$) and, consequently, is not at all representative of the State of São Paulo retail market. It was believed that the samples from "fish and pay" establishments would be more susceptible to the presence of contaminants because these establishments breed fishes of many species in a single tank, normally using antibiotics to prevent the spread of bacterial diseases between them, and these establishments are rarely inspected by sanitary vigilance agencies. It was also expected that samples from formal commercial establishments, such as supermarkets, would not present contaminant residues. Further investigation is necessary to confirm these expectations.

4. Conclusions

The developed method was suitable for the identification and quantification of macrolides (erythromycin, josamycin, tilmicosin, tylosin, spiramycin) in tilapia fillet. The single-laboratory validation results were satisfactory considering the recommendations of EC (2002), MAPA (2009) and IUPAC (Thompson et al., 2002) validation guides.

The extraction and clean up procedures were simple and less expensive than other methods published in scientific literature with the same purpose, and resulted in adequate recoveries and precision.

Matrix effect was significant ($\alpha = 5\%$) for all analytes except tylosin, which demonstrates the importance of matrix matched calibration when atmospheric pressure ionization techniques (such as ESI) are applied in mass spectrometry analysis. The use of a mass spectrometer able to get full scan mass spectra was important to clarify the matrix effect mechanism, through the difference of sodium adduct ions and protonated molecules ratios in the presence and absence of the matrix. This phenomenon, to our knowledge, has not yet been published.

The use of the LC–QToF technique allowed the acquisition of full scan spectra with good sensitivity and exact mass measurements, presenting advantages on analyte identification when compared to other MS configurations such as triple quadrupole, which is the most commonly applied technique for the quantification of contaminants in food matrices.

The limits of detection (LOD) and quantification (LOQ) proved that the developed method is adequate for the quantification and identification of the analytes in tilapia fillet samples at lower concentrations than the adopted MRLs. The difference between CCαs and MRLs were lower than 10% of the MRL, corroborating the method precision and its ability for application in regulatory analysis.

The validated method was applied to analyze tilapia samples available in the retail market in São Paulo State, Brazil, and no detectable levels of macrolide residues were found in these samples.

Acknowledgements

The authors gratefully acknowledge the financial support received from grants # 2008/00456-6, 2011/07963-3 and 04/09149-8, São Paulo Research Foundation (FAPESP); the Coordination for the Improvement of Higher Level Education Personnel (CAPES – Process No. 23038.003966/2013-08); and the National Council for Scientific and Technological Development (CNPq – Process No. 475492/2008-0 and 303670/2010-0), Brazil.

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